time scale: the microsecond time scale by $T_{1\rho}$ and the nanosecond time scale by the pre-steady-state NOE. NOE data indicate that B-d$(CG)_3$ is more mobile than Z-d$(CG)_3$ in 2 M $NaClO_4$ on the nanosecond time scale. However, $T_{1\rho}$ data indicate that the reverse is true on the microsecond time scale. Light-scattering experiments[26] and NMR proton-exchange studies[27] indicate that the B form is more mobile than the Z form and support our NOE results.

It is evident from our $T_{1\rho}$ and NOE results that B- and Z-d$(CG)_3$ each have different internal mobilities on different time scales. To our knowledge, this is the first observation that the degree of the internal mobilities of biological macromolecules is reversed in different time domains.

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Histidinol Dehydrogenase: ^{18}O Isotope Shift in ^{13}C NMR Reveals the Origin of Histidine Oxygens

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Abstract: The reaction catalyzed by L-histidinol dehydrogenase (EC 1.1.1.23) is the unusual NAD-linked 4-electron oxidation of L-histidinol carried out by way of an unknown aldehyde level intermediate that is covalently linked to the enzyme. Proposals have included an imine derivative of an active site lysine. The ^{18}O isotope shift in ^{13}C NMR was used to investigate the origin of the two oxygens in product histidine. Histidinol dehydrogenase from *Salmonella typhimurium* was incubated with highly enriched L-[hydroxymethyl-^{13}C]histidinol with NAD in 50% $H^{18}OH$; an NAD regenerating system was used to maintain high levels of NAD. Direct examination of the product L-[carboxy-^{13}C]histidine in ^{13}C NMR revealed two peaks separated by 0.02 ppm, indicative of incorporation of a single solvent oxygen. The failure to expel the original histidinol oxygen provides evidence against the participation of an imine in the reaction pathway.

The reaction catalyzed by L-histidinol dehydrogenase (HDH; EC 1.1.1.23) is a 4-electron oxidation of the amino alcohol substrate by two molecules of NAD to produce histidine, the final step in the bacterial,[1] fungal,[2] and plant[3] biosyntheses of this amino acid. The enzyme from *Salmonella typhimurium*[4] has been most widely studied. The key mechanistic problem posed by HDH is the chemistry of the aldehyde-level intermediate. Here, we use the ^{18}O perturbation of ^{13}C chemical shifts[5] to find that product histidine contains only a single solvent oxygen, demonstrating that formation of the intermediate does not expel the original histidinol oxygen, and ruling out an intermediate imine.

Externally added synthetic histidinaldehyde is chemically and kinetically competent to be the partially oxidized intermediate in the HDH reaction; however, during the overall oxidation of histidinol, histidinaldehyde is neither released to the medium nor accessible to aldehyde derivatizing reagents.[1] Gorisch and Holke[6] have shown that added histidinaldehyde is bound very tightly to HDH ($K_D = 1.4 \times 10^{-11}$ M), with $k_{off} = 2.6 \times 10^{-5}$ s^{-1}, and is protected in its bound form from degradation by solvent. These observations have led to the hypothesis that the intermediate is covalently bound to the protein.[1,6,7] Several proposals have been made[6-8] that an imine derivative of an active site lysine occurs, as demonstrated for uridine 1,6-diphosphoglucose dehydrogenase (UDPGDH, EC 1.1.1.22), another 4-electron dehydrogenase;[9] a thiohemiacetal has also been suggested for both enzymes.[7,8,10] The imine intermediate is attractive given the ease of formation and analogy with UDPGDH; however, the chemistry of an imine intermediate does not suggest a mechanistic route for the second oxidation. Chemically, a thiohemiacetal provides a plausible route for an NAD-linked oxidation to a thiolester, followed by hydrolytic cleavage to produce the product acid. The mechanism of the aldehyde oxidation step is of general interest, since aldehyde dehydrogenases are thought in general to use thiohemiacetals, although cysteine modification of human mitochondrial aldehyde dehydrogenase does not abolish activity.[11]

As shown in Scheme I, formation of the imine of histidinaldehyde would expel the original oxygen atom of histidinol leading to histidine product containing two solvent oxygens; the thiohemiacetal mechanism does not predict loss of the histidinol oxygen. Thus, by performing the HDH reaction in $H^{18}OH$ and analyzing the isotopic composition of the product one can distinguish these possibilities. Such oxygen expulsion has been observed in the case of fructose-1,6-phosphate aldolase.[12] The laboratory of Van Etten has detailed the upfield isotope shift caused by ^{18}O on the NMR chemical shift of adjacent ^{13}C atoms,[5] an effect that is additive in multiply substituted carbon atoms.[13] The same group has also demonstrated that this technology can be used to study reaction pathways.[14] Here, we investigated the

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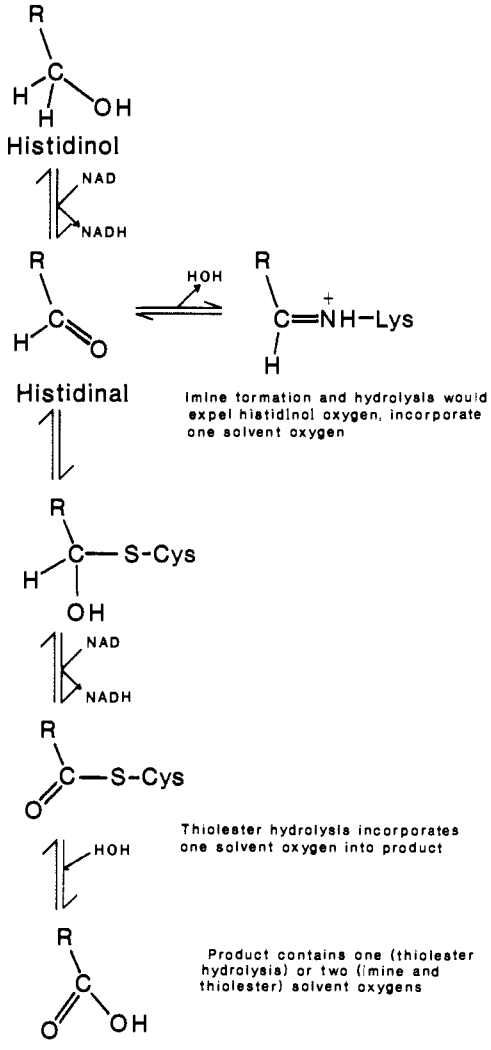
Scheme I. Alternative Routes for Incorporation of Solvent Oxygen by the HDH Reaction



^{13}C NMR of the carboxyl carbon of histidine produced by HDH-catalyzed oxidation of histidinol in $H^{18}OH$ and have found retention of the original histidinol oxygen, providing strong evidence against the existence of an imine intermediate in the HDH reaction.

Materials and Methods

The experiment was performed with 97% enriched L-[hydroxymethyl-^{13}C]histidinol. This compound was prepared from L-[carboxy-^{13}C]histidine (MSD Isotopes; thin-layer chromatography, ^{13}C NMR and ^{1}H NMR were employed to assess the purity of the compound) by esterification, reduction, and purification as described elsewhere;[15] identification of product histidinol was by enzymatic assay and thin-layer chromatography. To generate the histidine required to acquire ^{13}C spectra of adequate resolution, we adopted the FMN regenerating system of Jones and Taylor.[16] The HDH reaction was performed in a sealed tube with 25 μmol of sodium glycine, 5 μmol of NAD, 5 μmol of FMN, and 25 μmol of labeled histidinol, in a final volume of 0.5 mL containing 47% $H^{18}OH$. In some experiments, $MnCl_2$ (0.1 μmol) was also present. Highly purified *Salmonella typhimurium* HDH[17] (100 μg) was added to start the reaction. The mixture was stirred at room temperature for 2–4 h, during which time its color changed quickly from red (FMN) to green ($FMNH_2$) and slowly back to red. The final color change correlated with complete conversion of histidinol to histidine, as monitored by chromatography on paper. No purification of the enriched product was required under these conditions. EDTA (50 μmol) was added to chelate residual or added Mn(II), and samples were dried in a Savant Speed-Vac,

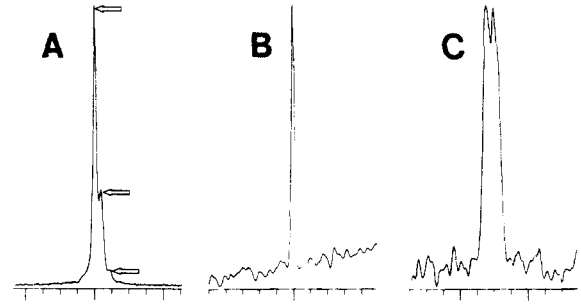


Figure 1. 75.5-MHz ^{13}C NMR spectra of carboxyl groups: (A) [1-^{13}C]acetate, equilibrated with 20% $H^{18}OH$; (B) [carboxy-^{13}C]histidine; (C) [carboxy-^{13}C]histidine produced by oxidation of ^{13}C-histidinol with HDH in 50% $H^{18}OH$. In each case one scale division is equivalent to 0.05 ppm, and the peaks are centered on 181.6 ppm (A) and 172.6 (B and C), relative to a standard of TMS.

redissolved in 0.5 mL of D_2O, and placed in 0.5-mm NMR tubes and ^{13}C NMR spectra recorded.

NMR spectra were collected on a General Electric GN-300 at a spectrometer frequency of 75.49 MHz with proton decoupling at 70 dB. A 7.5-μs pulse with a 5-s delay between pulses was employed. Initial spectra (256 scans) were collected over a spectral range of 19000 Hz to a data set of 32000 points. Subsequent spectra (512 scans) were collected to the same size data set with a 3000-Hz range centered around the peak of interest. This produced a resolution of more than 10 points/Hz. Data were Gaussian apodized introducing 0.8 Hz of line broadening.

Results and Discussion

Experiments were first performed to visualize non-enzymatic solvent equilibration of the carboxyl group. In one case, [1-^{13}C]acetate was equilibrated at 60 °C for 12 h in dilute HCl, pH 2.0, containing 20% $H^{18}OH$. The spectrum of this compound was recorded in Figure 1A. Three peaks are observed (arrows), separated by 0.025 ppm, as noted for ^{18}O chemical shifts in other carboxylic acids. The peaks represent $^{13}C^{16}O_2$, $^{13}C^{16}O_1{}^{18}O_1$, and $^{13}C^{18}O_2$ and are present in the 64:32:4 ratio expected for equilibration of both carboxyl oxygens with solvent. In a control experiment, [carboxy-^{13}C]histidine was exposed to the HDH reaction conditions described above for 24 h. No equilibration of the carboxy group oxygens with solvent was observed (Figure 1B).

A ^{13}C spectrum of histidine produced via the HDH reaction on ^{13}C-enriched histidinol in 50% ^{18}O water is shown in Figure 1C. Rather than the three peaks (25:50:25) expected if both carboxyl oxygens arose from solvent, two peaks (50:50) are observed, indicating a single oxygen has come from solvent. HDH is a Zn(II) metalloenzyme and is both stimulated and stabilized when Mn(II) is added to the medium;[18] this addition had no effect on the results of the experiment. We have also performed the experiment using natural abundance histidinol using a scaleup of the reaction conditions above and purifying the product histidine by ion exchange. Essentially the same result was observed, but spectral integrations suggested that some loss of ^{18}O had occurred, presumably catalyzed by the cation exchange resin.

It is thus clear that histidine produced by the HDH reaction contains only a single solvent oxygen, as well as the oxygen atom originating from histidinol. This observation is incompatible with the proposal of an imine intermediate, which involves expulsion of the substrate oxygen.

The loss of an imine intermediate as a viable step in catalysis has important implications for the structural basis of HDH catalysis. On the basis of the unusual genetics of the *hisD* locus of *Salmonella typhimurium*, and interesting observations made on partially denatured enzyme,[7] it has been suggested that HDH is "bifunctional" in that two structurally distinct subsites exist to perform the alcohol and aldehyde level oxidations. It has further been suggested that these subsites are shared between the two

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subunits of the dimer and that a lysine might serve as an "arm" to transfer the aldehyde equivalent as its imine between the two subsites. We note that results of biochemical characterization[4] and in vitro complementation studies[19] on isolated mutant subunits do not provide support for this hypothesis in its simplest form and that catalysis is more readily explained by a single active site oxidizing both the alcohol substrate and a structurally similar thiohemiacetal, hemiacetal, carbinolamine, or hydrate.

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Our knowledge of aldehyde oxidations is rather poor, being based on GAPDH, which uses a thiohemiacetal, and enzymes like glucose-6-phosphate dehydrogenase that employ substrates in which the aldehyde group is present as a readily oxidized derivative. Explorations of the chemistry of HDH may reveal new paradigms for NAD-linked enzymatic oxidation.

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An Unexpected Cis Peptide Bond in the Minor Conformation of a Cyclic Hexapeptide Containing Only Secondary Amide Bonds†

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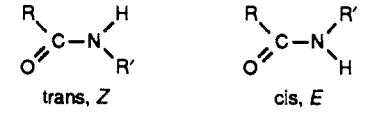
Abstract: The conformation of the cyclic hexapeptide *cyclo*(-D-Ala[1]-Phe[2]-Val[3]-Lys(Z)[4]-Trp[5]-Phe[6]-) [VDA008], a very potent inhibitor of the bile-acid transport system in hepatocytes, was investigated in hexadeuteriodimethyl sulfoxide solution by NMR spectroscopy and restraint MD calculations. Surprisingly, this peptide exhibits two conformations (94:6) in slow exchange on the NMR time scale at room temperature. Chemical exchange between both isomers was proven by 2D-ROESY and 2D-NOESY spectra. The dominant conformation has all-trans peptide bonds forming a βII,βII′ backbone conformation and preferred side chain orientations similar to that of the analogue *cyclo*(-D-Pro-Phe-Thr-Lys(Z)-Trp-Phe-), 008, whereas the minor conformation has a cis peptide bond between Lys(Z)[4] and Trp[5] forming a βVI turn about these residues. The conformational analyses of the backbones of both conformers are based on the temperature dependences of H_N chemical shifts, on scalar coupling constants, and on quantitative evaluation of ROEs and NOEs for restraint MD calculations. The populations of the side chain rotamers are derived from quantitative evaluation of homonuclear coupling constants and qualitative evaluation of heteronuclear coupling constants. For the MD calculations in vacuo, the side chains are fixed in the dominant rotamers.

Introduction

The cyclic hexapeptide *cyclo*(-D-Ala[1]-Phe[2]-Val[3]-Lys(Z)[4]-Trp[5]-Phe[6]-) [VDA008] was synthesized in the course of structure–activity studies of a number of cyclic hexapeptides, originally derived from somatostatin and antamanide, as inhibitors of a hepatocytic transport system.[1] Usually only one amino acid was replaced at a time, but a comparison of various analogues suggested that replacement of D-Pro[1] in "008"[1h] by D-Ala and Thr[3] by Val would be advantageous. In fact, VDA008 turned out to be the most potent cyclic hexapeptide in the biological tests, exceeding the activity of 008 by more than an order of magnitude.[2]

During NMR studies of this peptide cross-peaks, unequivocally indicating a slow chemical exchange between two conformers (relative population 94:6), were found in ROESY and NOESY spectra. This study revealed that the conformers are trans–cis isomers about the Lys(Z)[4]–Trp[5] peptide bond. Cis–trans isomerism about the amide bond was the first problem studied by dynamic NMR spectroscopy.[3,4] It has been shown that the barrier of amide bond rotation is in the order of 80 kJ/mol.[5-9] In secondary N-alkylamides the trans configuration is generally strongly preferred in solution;[5-9] cis configurations have so far only been observed in *N*-methylacetamide (1–3%),[10] ortho-substituted



acetanilides,[11] strained cyclic structures,[12] and in a small protein in micellar solution.[13] On the contrary, the occurrence of cis-

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† Abbreviations: E. COSY, exclusive correlated spectroscopy; DEPT-H,C-COSY, distorsionless enhancement by polarization transfer heteronuclear correlated spectroscopy; DQF-H,H-COSY, double quantum filtered proton correlated spectroscopy; H,C-COLOC, heteronuclear correlation via long-range couplings; NOE, nuclear Overhauser effect/enhancement; NOESY-nuclear Overhauser and exchange spectroscopy; ROE, rotating frame NOE; ROESY, rotating frame Overhauser and exchange spectroscopy, TOCSY, total correlation spectroscopy; Boc *tert*-butoxycarbonyl; Z, benzyloxycarbonyl.

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